SPHINGOID POLYALKYLAMINE CONJUGATES FOR VACCINATION

FIELD OF THE INVENTION

The present invention concerns vaccination making use of sphingolipids' polyalkylamine conjugates for effective delivery of biologically active materials, in particular, antigenic molecules.

LIST OF PRIOR ART

The following is a list of prior art which is considered to be pertinent for describing the state of the art in the field of the invention.

US 5,334,761: "Cationic lipids";

US 2001/048939: "Cationic reagents of transfection";

US 5,659,011: "Agents having high nitrogen content and high cationic charge based on dicyanimide dicyandiamide or guanidine and inorganic ammonium salts";

US 5,674,908: "Highly packed polycationic ammonium, sulfonium and phosphonium lipids";

US 6,281,371: "Lipopolyamines, and the preparation and use thereof";

US 6,075,012: "Reagents for intracellular delivery of macromolecules";

US 5,783,565: "Cationic amphiphiles containing spermine or spermidine cationic group for intracellular delivery of therapeutic molecules";

Marc Antoniu Ilies & Alexandru T. Balaban, Expert Opin. Ther. Patents. 11(11):1729-1752 (2001);

Miller AD. Chem. Int. Ed. Eng. **37**:1768-1785 (1998);

Nakanichi T. et al. J. Control Release **61**:233-240 (1999);

Brunel F. et al. Vaccine **17**:2192-2193 (1999);

Guy B. et al. Vaccine **19**:1794-1805 (2001);

Lima KM et al. Vaccine **19**:3518-3525 (2001).

BACKGROUND OF THE INVENTION

Many natural biological molecules and their analogues, including proteins and polynucleotides, foreign substances and drugs, which are capable of influencing cell function at the sub-cellular or molecular level are preferably incorporated within the cell in order to produce their effect. For these agents the cell membrane presents a selective barrier which is impermeable to them. The complex composition of the cell membrane comprises phospholipids, glycolipids, and cholesterol, as well as intrinsic and extrinsic proteins, and its functions are influenced by cytoplasmic components which include Ca^{++} and other metal ions, anions, ATP, microfilaments, microtubules, enzymes, and Ca^{++}-binding proteins, also by the extracellular glycocalyx (proteoglycans, glycose aminoglycans and glycoproteins). Interactions among structural and cytoplasmic cell components and their response to external signals make up transport processes responsible for the membrane selectivity exhibited within and among cell types.

Successful delivery of agents not naturally taken up by cells into cells has also been investigated. The membrane barrier can be overcome by associating agents in complexes with lipid formulations closely resembling the lipid composition of natural cell membranes. These formulations may fuse with the cell membranes on contact, or what is more common, taken up by pynocytosis, endocytosis and/or phagocytosis. In all these processes, the associated substances are delivered in to the cells.

Lipid complexes can facilitate intracellular transfers also by overcoming charge repulsions between the cell surface, which in most cases is negatively charged. The lipids of the formulations comprise an amphipathic lipid, such as the phospholipids of cell membranes, and form various layers or aggregates such as micelles or hollow lipid vesicles (liposomes), in aqueous systems. The liposomes can be used to entrap the substance to be delivered within the liposomes; in other applications, the drug molecule of interest can be incorporated into the lipid vesicle as an intrinsic membrane component, rather than entrapped into the hollow aqueous interior, or electrostatically attached to

aggregate surface. However, most phospholipids used are either zwiterionic (neutral) or negatively charged.

An advance in the area of intracellular delivery was the discovery that a positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), in the form of liposomes, or small vesicles, could interact spontaneously with DNA to form lipid-DNA complexes which are capable of adsorbing to cell membranes and being taken up by the cells either by fusion or more probably by adsorptive endocytosis, resulting in expression of the transgene [Felgner, P. L. et al. Proc. Natl. Acad. Sci., USA 84:7413-7417 (1987) and U.S. Pat. No. 4,897,355 to Eppstein, D. *et al.*]. Others have successfully used a DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) in combination with a phospholipid to form DNA-complexing vesicles. The Lipofectin™ reagent (Bethesda Research Laboratories, Gaithersburg, MD.), an effective agent for the delivery of highly anionic polynucleotides into living tissue culture cells, comprises positively charged liposomes composed of positively charged lipid DOTMA and a neutral lipid dioleyl phosphatidyl ethanol amine (DOPE) referred to as helper lipids. These liposomes interactspontaneously with negatively charged nucleic acids to form complexes, referred to as lipoplexes. When excess of positively charged liposomes over DNA negative charges are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces or introduced into the cells either by adsorptive endocytosis or fuse with the plasma membrane, both processes deliver functional polynucleotide into, for example, tissue culture cells. DOTMA and DOTAP are good examples for monocationic lipids. [Illis et al. 2001, ibid.]

Multivalent cations by themselves (including polyamines, inorganic salts and complexes and dehydrating solvents) have also been shown to facilitate delivery of macromolecules into cells. In particular, multivalent cations provoke the collapse of oligo and polyanions (nucleic acids molecules, amino acid molecules and the like) to compact structural forms, and facilitate the packaging

of these polyanions into viruses, their incorporation into liposomes, transfer into cells etc. [Thomas T.J. et al. Biochemistry 38:3821-3830 (1999)]. The smallest natural polycations able to compact DNA are the polyamines spermidine and spermine. By attaching a hydrophobic anchor to these molecules via a linker, a new class of transfection vectors, the polycationic lipopolymers, has been developed.

Cationic lipids and cationic polymers interact electrostatically with the anionic groups of DNA (or of any other polyanionic macromolecule) forming DNA-lipid complexes (lipoplexes) or DNA-polycation complexes (polyplexes). The formation of the complex is associated with the release of counterions of the lipids or polymer, which is the thermodynamic driving force for lipoplex and polyplex spontaneous formation. The cationic lipids can be divided into four classes: (i) quaternary ammonium salt lipids (e.g. DOTMA (Lipofectin™) and DOTAP) and phosphonium/arsonium congeners; (ii) lipopolyamines; (iii) cationic lipids bearing both quaternary ammonium and polyamine moieties and (iv) amidinium, guanidinium and heterocyclic salt lipids.

SUMMARY OF THE INVENTION

According to one of its aspects, the present invention concerns the use of a sphingoid-polyalkylamine conjugate for the preparation of a pharmaceutical composition for modulating the immune response of a subject.

According to a preferred embodiment, the sphingoid-polyalkylamine conjugate comprises a sphingoid backbone carrying, via a carbamoyl bond at least one polyalkylamine chain polyalkylamine chain.

The term ***sphingoid-polyalkylamine conjugate*** as used herein denotes chemical conjugation (linkage) between a sphingoid base (herein also referred to by the term "sphingoid backbone") and at least one polyalkylamine chain. The conjugation between the sphingoid base and the at least one polyalkylamine chain is via a carbamoyl bond, as further detailed hereinafter.

The ***sphingoid base/backbone***, as used herein, includes, long chain aliphatic amines, containing two or three hydroxyl groups, the aliphatic chain may be saturated or unsaturated. One example of an unsaturated sphingoid base is that containing a distinctive trans-double bond in position 4.

The term ***modulating*** as used herein denotes any measurable regulatory or biochemical effect exhibited by the biologically active material delivered by the conjugate, on a subject's immune response, including cellular response and/or humoral response. Modulation includes inhibition or, on the other hand, stimulation or enhancement of either or both types of responses when the sphingoid-polyalkylamine conjugate is administered to said subject in combination with a biologically active substance. The modulation preferably refers to stimulation or enhancement by a factor of two or more, relative to that elicited by the biological active molecule administered without the conjugate. The invention also concerns the modulation of an immune response in cases when the biologically active material administered without the conjugate is substantially ineffective in producing such a response.

Yet further, modulation concerns inhibition or suppression of the immune response of a subject, e.g. for the treatment of auto-immune diseases as well as for the treatment of allergy.

Thus, the term ***biologically active molecule*** as used herein denotes any substance which, when administered in combination with the sphingoid-polyalkylamine conjugate has an effect on the immune system of a subject. The biologically active material is preferably an antigenic protein, antigenic peptide, antigenic polypeptide or antigenic carbohydrate.

According to another aspect, the present invention concerns a method for modulating the immune response of a subject, the method comprises providing said subject with a sphingoid-polyalkylamine conjugate together with a biologically active molecule, said sphingoid-polyalkylamine conjugate comprises a sphingoid backbone carrying, via a carbamoyl bond at least one polyalkylamine chain.

According to yet another aspect, the present invention concerns a pharmaceutical composition for modulating the immune response of a subject, the composition comprises: (i) at least one sphingoid-polyalkylamine conjugate; and (ii) at least one biologically active molecule associated with said conjugate.

According to yet another embodiment, the invention provides a complex comprising: (i) a sphingoid-polyalkylamine conjugate and (ii) a biologically active material capable of modulating an immune response of a subject.

Finally, the invention concerns the use of

Finally, the invention concerns the use of a sphingoid-polyalkylamine conjugate as defined, as a capturing agent of biologically active molecules (e.g. antigenic molecules). In this context, the sphingoid-polyalkylamine conjugate may form part of a kit for capturing biologically active molecules, preferably antigenic molecules and/or immunostimulants, and/or immunosuppressants, the kit comprising, in addition to said conjugate, instructions for use of same for capturing the biologically active molecules. The conjugate in the kit may be in a dry form, in which case, the kit may also include a suitable fluid with which the conjugate is mixed prior to use to form a suspension or emulsion or solution, or it may already be in a fluid (suspension, emulsion, solution, etc.) form. The kit may have numerous applications. For example, the kit may be used for investigating the function of different immunomodulating molecules in modulation of immune responses, for isolation of active biological molecules, and identification thereof. Those versed in the art will know how to make use of such a capturing agent also for research purposes.

The term ***capturing agent*** as used herein refers to the conjugate being capable of associating with biologically active molecules, the latter having a negative charge, a negative dipole or a local negative charge (an area within the molecule carrying a net negative charge), by virtue of the conjugate's polycationic structure. The capturing per se involves electrostatic interaction between the molecule to be captured, carrying said negative charge, negative dipole or local negative charge and the positively charged conjugate of the invention.

The conjugate of the invention may also be used as a delivery vehicle, carrying, by capturing thereto, biologically active molecules to a target site and into a target cell.

BRIEF DESCRIPTION OF THE FIGURES

In order to understand the invention and to see how it may be carried out in practice, some embodiments will now be described, by way of non-limiting examples only, with reference to the accompanying figures, in which:

Figs. 1A-1D show several possible chemical structures, "linear", branched" or "cyclic" lipid like cationic (LLC) compounds which are encompass under the general definition of sphingoid- polyalkylamine conjugate of formula (I), wherein **Fig. 1A** shows a sphingoid backbone (ceramide) linked to a single polyalkylamine chain, **Fig. 1B** and **Fig. 1C** show the same sphingoid backbone linked to two polyalkylamine chains, **Fig. 1D** shows again the same backbone, however, in which a single polyalkylamine chain is linked via the two hydroxyl moieties to form a cyclic polyalkylamine conjugate.

Figs. 2A-2F show the bio-distribution and pharmacokinetics of various fluorescently-labeled lipid formulations in the GI-■-, lungs -◆- or spleen --- with unrecovered -◈-: **Fig 2A** shows distribution of empty DMPC:DMPG (mole ratio 9:1); **Fig. 2B** shows distribution of DMPC:DMPG:HN; **Fig. 2C** shows distribution of empty DOTAP:cholesterol; **Fig. 2D** shows distribution of DOTAP:cholesterol:HN; **Fig. 2E** shows distribution of empty CCS:cholesterol; and finally, **Fig. 2F** shows distribution of CCS-cholesterol:HN.

Figs. 3A-3D show bio-distribution of various ^{125}I-HN loaded lipid assembly formulations in the GI-■-, lungs -◆- or spleen -◈- with unrecovered -x-, and in particular, **Fig. 3A** shows bio-distribution of free ^{125}I-HN; **Fig. 3B** shows ^{125}I-HN loaded lipid assembly composed of DOTAP:Cholesterol; **Fig. 3C** shows ^{125}I-HN loaded lipid assembly composed of DMPC:DMPG and **Fig. 3D** shows ^{125}I-HN loaded lipid assembly composed of CCS:Cholesterol.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns the use of sphingoid-polyalkylamine conjugates as capturing agents for carrying biologically active molecules which are effective in modulating the immune response of a subject.

The sphingoid-polyalkylamine conjugates are lipid-like cationic (LLC) compounds, which may be synthesized in the following manner. N-substituted long-chain bases in particular, N-substituted sphingoids or sphingoid bases are coupled together with different polyalkylamines or their derivatives, to form a polyalkylamine-sphingoid entity, which is used as is, or further alkylated.

Protonation at a suitable pH or alkylation of the formed polyalkylamine-sphingoid entity attributes to the lipid-like compounds a desired positive charge for interaction with biologically active biological molecules to be delivered into target cells and with the targeted cells. The sphingoid-polyalkylamine conjugates may be efficiently associated with the biologically active molecules by virtue of electrostatic interactions between the anionic character of the biologically active molecules and the polyalkylamine moieties of the conjugate to form complexes (lipoplexes).

Alternatively, the sphingoid-polyalkylamine conjugates may form assemblies loaded with the biologically active molecules.

The sphingoid-polyalkylamine conjugate may be in the form individual lipid like molecules or in the form of an assembly. One example of a suitable assembly includes the formation of micelles or vesicles, and in particular, liposomes. Other examples of assemblies include the formation of micelles, inverted phases, cubic phases and the like. Evidently, the sphingoid polyalkylamine conjugate may be in combined vesicle/micelle form or any other combination of assemblies.

Lipid assembly as used herein denotes an organized collection of lipid molecules forming *inter alia*, micelles and liposomes. The lipid assemblies are preferably stable lipid assemblies. ***Stable lipid assembly*** as used herein denotes an

assembly being chemically and physically stable under storage conditions (4°C, in physiological medium) for at least one month.

When the assemblies are in the form of vesicles (e.g. liposomes) the biologically active molecule may be encapsulated within the vesicle, part of its lipid bilayer, or adsorbed to the surface of the vesicle (or any combination of these three options). When the assemblies are micelles, the biologically active molecules may be inserted into the amphiphiles forming the micelles and/or associated with it electrostatically, in a stable way.

Thus, as used herein, the terms "***encapsulated in***", "***contained in***", "***loaded onto***" or "***associated with***" indicate a physical attachment between the conjugate and the biologically active molecule. The physical attachment may be either containment or entrapment of the molecule within assemblies (e.g. (vesicles, micelles or other assemblies) formed from the conjugate; non-covalent linkage of the biological molecule to the surface of such assemblies, or embedment of the biological molecule in between the sphingoid-polyalkylamine conjugates forming such assemblies. It should be noted that due to the positive charge or positive dipole of the sphingoid-polyalkylamine conjugate under physiological conditions, the preferred association between the conjugate and the biologically active material is by electrostatic, dipole or acid-base interactions.

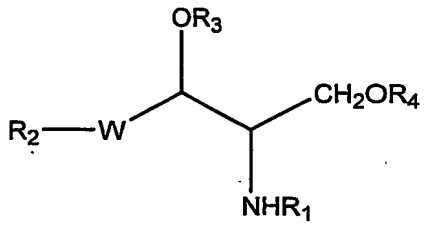
Notwithstanding the above, the invention should not be limited by the particular type of association formed between the sphingoid-polyalkylamine conjugate and the biologically active molecule. Thus, association means any interaction between the conjugate or the assembly formed therefrom and the biologically active material which is capable of achieving a desired therapeutic effect.

The biologically active molecule and the conjugate may be associated by any method known in the art. This includes, without being limited thereto, post- or co-lyophilzation of the conjugate with the biologically active molecule, or by mere mixing of preformed sphingoid-polyalkylamine conjugate with the biological molecule. Method for co-lyophilization are described, *inter alia*, in

U.S. patent Nos. 6,156, 337 and 6,066,331, while methods for post-encapsulation are described, *inter alia*, in WO03/000227, all incorporated herein by reference.

Thus, according to a first of its aspects, the present invention concerns the use of a sphingoid-polyalkylamine conjugate for the preparation of a pharmaceutical composition for modulating the immune response of a subject, wherein said sphingoid-polyalkylamine conjugate comprises a sphingoid backbone carrying, via a carbamoyl bond, at least one, and preferably one or two, polyalkylamine chains.

As indicated above, the sphingoid-polyalkylamine conjugate includes a linkage between a sphingoid backbone and at least one polyalkylamine chain, the linkage is via corresponding carbamoyl bonds. More preferably, the sphingoid-polyalkylamine conjugate has the general formula (I):



wherein

$\mathbf{R_1}$ represents a hydrogen, a branched or linear alkyl, aryl, alkylamine, or a group -C(O)R_5;

$\mathbf{R_2}$ and $\mathbf{R_5}$ represent, independently, a branched or linear C_{10}-C_{24} alkyl, alkenyl or polyenyl groups;

$\mathbf{R_3}$ and $\mathbf{R_4}$ are independently a group -C(O)-NR_6R_7, $\mathbf{R_6}$ and $\mathbf{R_7}$ being the same or different for R_3 and R_4 and represent, independently, a hydrogen, or a saturated or unsaturated branched or linear polyalkylamine, wherein one or more amine units in said polyalkylamine may be a quaternary ammonium; or

$\mathbf{R_3}$ is a hydrogen; or

$\mathbf{R_3}$ and $\mathbf{R_4}$ form together with the oxygen atoms to which they are bound a heterocyclic ring comprising –C(O)-NR_9–$[R_8$–$NR_9]_m$-C(O)–, $\mathbf{R_8}$ represents a saturated or unsaturated C_1-C_4 alkyl and $\mathbf{R_9}$ represents a hydrogen or a

polyalkylamine of the formula $-[R_8-NR_9]_n-$, wherein said R_9 or each alkylamine unit R_8NR_9 may be the same or different in said polyalkylamine; and

n and **m** are independently an integer from 1 to 10, preferably 3 to 6;

W represents a group selected from $-CH=CH-$, $-CH_2-CH(OH)-$ or $-CH_2-CH_2-$.

Non-limiting examples of the sphingoids or sphingoid bases which may be used according to a more specific embodiment of the invention, include, sphingosines, dihydrosphingosines, phytosphingosines, dehydrophytosphinosine and derivatives thereof. Non-limiting examples of such derivatives are acyl derivatives, such as ceramide (N-acylsphingosine), dihydroceramides, phytoceramides and dihydrophytoceramides, respectively, as well as ceramines (N-alkylsphinogsine) and the corresponding derivatives (e.g. dihydroceramine, phytoceramine etc.). The suitably N-substituted sphingoids or sphingoid bases posses free hydroxyl groups which are activated and subsequently reacted with the polyalkylamines to form the polyalkylamine-sphingoid entity. Non-limiting examples of activation agents are N,N'-disuccinimidylcarbonate, di- or tri-phosgene or imidazole derivatives. The reaction of these activation agents with the sphingoids or the sphingoid bases yields a succinimidyloxycarbonyl, chloroformate or imidazole carbamate, respectively, at one or both hydroxyls. The reaction of the activated sphingoids with polyalkylamines may yield branched, straight (unbranched) or cyclic conjugates as shown in Fig. 1.

According to one preferred embodiment the sphingoid backbone is a ceramide linked to one (Fig. 1A) or two (Fig. 1B or 1C) polyalkylamine chains, or linked via the two hydroxyl moieties to form a cyclic polyalkylamine moiety (Fig. 1D).

The formed sphingoid-polyalkylamine conjugates may be further reacted with methylation agents in order to form quaternary amines. The resulting compounds are positively charged to a different degree depending on the ratio between the quaternary, primary and/or secondary amines within the formed conjugates. As such, the sphingoid-polyalkylamine conjugate exists as quaternized nitrogen salt including, but not limited to, quaternary ammonium

chloride, a quaternary ammonium iodide, a quaternary ammonium fluoride, a quaternary ammonium bromide, a quaternary ammonium oxyanion and a combination thereof.

The sphingoid-polyalkylamine conjugate is preferably used in combination with a biologically active molecule. The biologically active material is any molecule which when administered with the sphingoid-polyalkylamine conjugate has an effect on the immune system of a subject, according to one embodiment, a stimulating or enhancing effect. The effect is preferably by a factor of two or more relative to the effect, if any, of the biologically active molecule, when provided to a subject without said conjugate.

According to one embodiment, the biologically active material is a protein, polypeptide, peptide, or carbohydrate. Specifically, the biologically active molecule may be an immunomodulator, including antigenic protein or antigenic peptide, immnostimulants and/or immunosuppressants. Antigenic proteins and peptides, immunostimulants and immunosuppressants are all well known in the art. Preferably, the biologically active protein or peptide or carbohydrate has at a physiological pH either a net negative dipole moment, a net negative charge or contains at least one region having a net negative charge negatively charged.

According to yet another embodiment, the biologically active material is a nucleic acid molecule, such as oligodeoxynucleotides (ODN).

A preferred weight ratio between the sphingoid-polyalkylamine conjugate and biologically active material is 1000:1 to 1:1 weight ratio.

The sphingoid- polyalkylamine conjugate may also be combined with other active substances known to be used in combination with antigenic molecules. Such substances include, for example, immunostimulating agents (also known by the term "immunostimulant" or "adjuvant"). This includes any substance that when added to a vaccine it improves the immune response so that less vaccine is needed to produce a greater response. The immunostimulating agent may be provided together with the conjugate/biologically active material, or

within a specified time interval (e.g. several hours or days before or after the administration of the conjugate/biologically active molecule).

Preferred immunostimulating agents include, without being limited thereto, cytokines, such as interleukins (IL-2, IL-10, IL-12, IL-15, IL-18), interferons (IFN alpha, beta, gamma), oligodeoxynucleotides (ODN), toxins (e.g. cholera toxin (CT), staphylococcal enterotoxin B (SEB)) heat label E. Coli enerotoxin (HLT)as well as any other adjuvants known to be used in the art for enhancing or stimulating the immune response to an antigenic molecule.

The assemblies may include the sphingoid-polyalkylamine conjugate (non-methylated or methylated) as the sole lipid-like ingredient, or be combined with other *helper* lipid substances. Such helper lipid substances may include non-cationic lipids like DOPE, DOPC, DMPC, Cholesterol, oleic acid or others at different mole ratios to the lipid-like compound. Cholesterol is one preferred added substance for in vivo application while DOPE may be a preferred helper lipid for in vitro applications. In this particular embodiment the mole ratio of cholesterol to cationic lipid is within the range of 0.01–1.0 and preferably 0.1–0.4.

The assemblies may also include enhancers (as known in the art, such as $CaCl_2$ and soluble polyalkylamines.

Other components which may be included in the lipid assembly, and which are known to be used in structures of the like, are steric stabilizers. One example of a commonly used steric stabilizer is the family of lipopolymers, e.g. polyethylene glycol derivatized lipids (PEG-lipid conjugate). This family of compounds are know, *inter alia*, to increase (extend) the circulation time of lipids.

According to one embodiment, the formed liposomes may be shaped as unsized heterogeneous and heterolamellar vesicles (UHV) having a diameter of about 50 – 5000 nm. The formed UHV, may be downsized and converted to large (more homogenous) unilamellar vesicles (LUV) having a diameter of about 50-100 nm by further processing. The structure and dimensions of the vesicles, e.g.

their shape and size may have important implications on their efficiency as vehicles for delivery of the active biological entities to the target, i.e. these determine their delivery properties.

A preferred group of polyalkylamine chains forming part of the sphingoid-polyalkylamine conjugate have been structurally defined hereinabove in connection with formula (I). According to this embodiment, the polyalkylamine chains, which may be the same or different in the conjugate of formula (I), are selected from spermine, spermidine, a polyalkylamine analog or a combination of same thereof. The term polyalkylamine analog is used to denote any polyalkylamine chain, and according to one embodiment denotes a polyalkylamine comprising 1 to 10 amine groups, preferably from 3- to 6 and more preferably 3 or 4 amine groups. Each alkylamine within the polyalkylamine chain may be the same or different and may be a primary, secondary, tertiary or quaternary amine.

The alkyl moiety, which may be the same or different within the polyalkylamine chain, is preferably a C_1-C_6 aliphatic repeating unit. Some non-limiting examples of polyalkylamine s include spermidine, N-(2-aminoethyl)-1,3-propane-diamine, 3,3`-iminobispropylamine, spermine and bis(ethyl) derivatives of spermine, polyethyleneimine.

The most preferred sphingoid-polyalkylamine conjugate according to the invention is N-palmitoyl D-erythro sphingosyl carbamoyl-spermine (CCS). This conjugate includes a ceramide linked via a carbamoyl bond to spermine.

The sphingoid-polyalkylamine conjugate according to the invention is preferably used for the preparation of a vaccine.

According to one embodiment, the sphingoid-polyalkylamine conjugate, and preferably the CCS, is used for the preparation of an influenza vaccine. In this particular embodiment, the biologically active material is derived from the influenza virus or a biologically active analog of a molecule derived from influenza virus. Such analogs include any substance which includes an influenza derived antigenic fragment which elicits an immune response.

A specific influenza derived antigenic material is the hemagglutinin (HA) and neuraminidase (NA) molecules, the combination is referred to as HN.

The present invention also concerns a method for modulating the immune response of a subject, the method comprises treating said subject with the sphingoid-polyalkylamine conjugate together with a biologically active material.

The combined treatment includes administration of the sphingoid-polyalkylamine conjugate and biologically active material either together, or within a predefined time interval, such as several hours or several days (optionally in combination with an immunostimulant). However, according to a preferred embodiment, the conjugate and biologically active material are mixed together prior to administration to the subject.

Administration of the sphingoid-polyalkylamine conjugate together with the biologically active material concerns another aspect of the invention. Accordingly, there is provided a pharmaceutical composition comprising a physiologically acceptable carrier and an effective amount of the sphingoid-polyalkylamine conjugate together with the biologically active material. The pharmaceutical composition optionally comprises an immunostimulant.

The sphingoid- polyalkylamine conjugated in combination with the biologically active material may be administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners.

The "***effective amount***" for purposes herein denotes an amount which is effective to modulate (enhance or stimulate, as defined above) the subject's immune response relative to the effect obtained when the biologically active material is provide to the subject without the sphingoid- polyalkylamine conjugate. Preferably, the amount is effective to achieve effective immunization of a subject against a specific disease or disorder.

Notwithstanding the above, the amount may be effective to achieve suppression or inhibition of the immune response, e.g. for the purpose of treating allergy or autoimmune responses.

The composition of the invention comprising the sphingoid-polyalkylamine conjugate associated with the biologically active material may be administered in various ways. Non-limiting examples of administration routes include oral, subcutaneous (s.c.), parenteral including intravenous (i.v.), intra-arterial (i.a.), intramuscular (i.m.), intraperitoneal (i.p.)' intrarectal (i.r.) and intranasal (i.n.) administration, as well as by infusion techniques to the eye intraocular. Preferably modes of administration are the intranasal or intramuscular administrations.

The physiologically acceptable carrier according to the invention generally refers to inert, non-toxic solid or liquid substances preferably not reacting with the biologically active material or with the conjugate and which is required for the effective delivery of the conjugate with the biologically active molecule.

Non-limiting examples of physiologically acceptable carrier include water, saline, 5% dextrose (glucose), 10% sucrose etc., either alone or with minor amounts (up to 10%) of an alcohol, such as ethanol.

Preferably, the composition of the invention is a liquid formulation, including suspensions, aqueous solutions or in the form of an aerosol, all of which are known to those versed in the art. Aerosol formulations can be placed into pressurized acceptable propellants, such as propane, nitrogen, and the like. They also may be formulated as pharmaceuticals for non-pressured preparations, such as in a nebulizer or atomizersuitable carriers.

DESCRIPTION OF THE SPECIFIC EXAMPLES

INFLUENZA

Characterization of HN Antigen-loaded cationic liposomes

Efficiency of encapsulation of HN (a commercial preparation of hemagglutinin and neuraminidase derived from influenza viruses) loaded onto

various cationic liposomal formulations, at different lipid/protein w/w ratios (3/1-300/1), and with or without cholesterol (Chol) was tested. Table 1 shows the results of such an experiment, using the cationic lipids DOTAP and CCS.

Table 1- The effect of the lipid (DOTAP, CCS)/protein ratio and cholesterol (Chol) on HN encapsulation efficiency

DOTAP/HN w/w ratio	DOTAP/Chol mole ratio	% HN encapsulation	CCS/HN w/w ratio	CCS/Chol mole ratio	% HN encapsulation
300/1	1/1	93	300/1	1/0	73
100/1	1/1	90	100/1	1/0	64
50/1	1/1	90	30/1	1/0	38
30/1	1/1	88	10/1	1/0	1
10/1	1/1	79			
3/1	1/1	35			
100/1	1/0	90	300/1	3/2	71
100/1	1/1	92	100/1	3/2	64
100/1	2/1	89	30/1	3/2	41
100/1	4/1	80	10/1	3/2	0

A monovalent vaccine was used for DOTAP and a trivalent vaccine for CCS.

The percentage of loading for DOTAP was 75-90% using a lipid/protein w/w ratio of 50/1 to 300/1, with and without Chol. At lipid/protein w/w ratios of 30/1 to 300/1, ~90% antigen loading was achieved, decreasing to 79% and 35% at 10/1 and 3/1 w/w ratios, respectively. The addition of Chol to the formulation did not affect loading at DOTAP/Chol mole ratios of 1/1 and 2/1, with slightly lower encapsulation (80%) at a ratio of 4/1. For CCS, with or without Chol, the loading efficiency was lower (64-73% at w/w ratio of 100/1-300/1).

HN association with the liposomes upon simple mixing of the soluble antigen with preformed empty liposomes was also determined. In such cases, 40-60% of the antigen was associated with the liposomes using a lipid/protein w/w ratio of 100/ to 300/1, regardless of the formulation.

These finding, collectively, indicate very high loading efficiency (>60%) using a simple and fast (5 min.) procedure in all formulations. Furthermore, even preformed liposomes in aqueous suspension were capable of effectively associating with the influenza virus surface antigens.

The immunogenicity of the various lipid/antigen w/w ratios (with or without the addition of cholesterol) was also evaluated.

In a first experiment sera levels of HI, IgG1 and IgG2a antibodies following i.n. vaccination of young (2-month-old) BALB/c mice with HN-loaded neutral, anionic or cationic liposomes were determined (Table 2A). The HN antigen was a monovalent subunit vaccine derived from the A/New Caledonia (H1N1) strain. In the same experiment, lung and nasal levels of HI, IgG1, IgG2a and IgA antibodies, and INFγ levels produced by spleen cells, were also tested (Tables 2B and 2C).

Table 2A – Sera levels of HI, IgG1 and IgG2a antibodies

Group (n=5)	Vaccine	Serum		
		HI (mean ± SD)	IgG1	IgG2a
1	PBS	0	0	0
2	F-HN	0	55	0
3	Lip (DMPC)-HN (Neutral)	3±7 (0)	150	0
4	Lip (DMPC/DMPG)-HN (Anionic)	6±13 (20)	500	0
5	Lip (DC-Chol:DOPE)-HN	18±7 (0)	0	0
6	Lip (DSTAP:Chol)-HN	28±29 (40)	20	0
7	Lip (DDAB: Chol)-HN	136±32 (100)	100	0
8	Lip (DOTAP: Chol)-HN	576±128 (100)	15000	730
9	Lip (DMTAP: Chol)-HN	672±212 (100)	30000	470
10	Lip (CCS: Chol)-HN	2368±1805 (100)	30000	9000
11	F-HN+CT (1μg)	1664±572 (100)	55000	7000

F-HN, free antigen; Chol, cholesterol; CT, cholera toxin. Groups 5-10 are cationic liposomes. In parentheses, % seroconversion - % of mice with HI titer ≥ 40.

In particular, a comparison was made between neutral (DMPC), anionic (DMPC/DMPG, 9/1 mole ratio) and cationic (6 formulations) assemblies (Lip) encapsulating the HN antigens to induce local and systemic responses following two i.n. administrations. For all formulations, the lipid/HN w/w ratio was 300/1, and the cationic lipid/Chol or cationic lipid/DOPE mole ratio was 1/1. Free antigen (F-HN) and F-HN co-administered with cholera toxin (CT, 1 μg) as an adjuvant were tested in parallel. The vaccine was given on days 0 and 7, 3

μg/dose (10 μl per nostril), and the responses were determined 4-6 weeks after the second vaccine dose.

Table 2B – lung and nasal wash levels of IgG1, IgG2a and IgA antibodies

Group (n=5)	Vaccine	Lung			Nasal		
		IgG1	IgG2a	IgA	IgG1	IgG2a	IgA
1	PBS	0	0	0	0	0	0
2	F-HN	0	0	0	0	0	0
3	Lip (DMPC)-HN (Neutral)	30	0	0	0	0	0
4	Lip (DMPC/DMPG)-HN (Anionic)	40	0	0	0	0	0
5	Lip (DC-Chol:DOPE)-HN	0	0	0	0	0	0
6	Lip (DSTAP:Chol)-HN	0	20	0	0	80	0
7	Lip (DDAB: Chol)-HN	0	80	30	0	30	0
8	Lip (DOTAP: Chol)-HN	730	1050	170	40	180	30
9	Lip (DMTAP: Chol)-HN	470	3000	30	15	80	70
10	Lip (CCS: Chol)-HN	9000	30000	1900	15000	30	300
11	F-HN+CT (1μg)	7000	10000	1800	40	120	30

Table 2C – Spleen INFγ levels (pg/ml)

Group (n=5)	Vaccine	Spleen
		IFNγ (pg/ml)
1	PBS	1800
2	F-HN	1400
3	Lip (DMPC)-HN (Neutral)	4200
4	Lip (DMPC/DMPG)-HN (Anionic)	4000
5	Lip (DC-Chol:DOPE)-HN	4900
6	Lip (DSTAP:Chol)-HN	2300
7	Lip (DDAB: Chol)-HN	3100
8	Lip (DOTAP: Chol)-HN	8000
9	Lip (DMTAP: Chol)-HN	7800
10	Lip (CCS: Chol)-HN	10200
11	F-HN+CT (1μg)	5200

As shown in **Tables 2A-2C**, the free antigen, as well as the neutral and anionic Lip-HN were virtually ineffective mucosal vaccines. In contrast, the cationic Lip-HN, particularly those designated DOTAP-HN, DMTAP-HN and CCS-HN evoked a robust systemic and mucosal humoral response, with high levels of IgG1, IgG2a and IgA antibodies, namely a mixed Th1+Th2 response. No IgE antibodies were defected. The cationic liposomal vaccines comprising DOTAP-HN, DMTAP-HN and CCS-HN also induced high levels of IFNγ (but

not IL-4) in antigen-stimulated spleen cells. The responses produced by CCS-HN were even stronger than those induced by F-HN adjuvanted with CT. Based on these findings, only the cationic liposomal formulations: DOTAP-HN, DMTAP-HN and CCS-HN were further used.

In a second experiment, the effect of lipid/HN w/w ratio on the immunogenecity of HN-loaded cationic liposomes and of preformed liposomes simply mixed with the soluble antigen, was determined. The data shown in **Tables 3A-3C** indicate that all three formulations induced a strong systemic (serum) and local (lung) response, and that lowering the lipid/HN w/w ratio below 100/1 markedly reduced the response.

Table 3A - Serum levels of HI, IgG1, IgG2a and IgA antibodies

No.	Vaccine (n=5)	Lipid/HN w/w ratio	Serum HI	IgG1	IgG2a	IgA
1	F-HN		0	0	0	0
2	Lip (DOTAP)-HN	300/1	496±295 (100)	15000	450	0
3		100/1	196±119 (100)	5000	280	0
4		30/1	36±50 (80)	1000	200	0
5		10/1	28±18 (60)	600	30	0
6		3/1	0	20	0	0
7	Lip (DMTAP)-HN	300/1	388±260 (100)	2500	250	0
8		100/1	208±107 (100)	2200	600	0
9		50/1	130±118 (80)	850	150	0
10		30/1	48±71 (40)	450	0	0
11		10/1	24±35 (40)	120	0	0
12	Lip (CCS)-HN	300/1	560±480 (100)	2000	1800	200
13		100/1	752±504 (100)	6500	6000	0
14		50/1	272±156 (100)	1900	700	0
15		30/1	112±125 (80)	650	400	0
16		10/1	52±68 (40)	275	440	0
17	F-HN+CT (1μg)	-	896±350 (100)	30000	8000	120
18	F-HN+Lip (DOTAP)	300/1	864±446 (100)	5000	1500	0
19	F-HN+Lip (DMTAP)	300/1	320±226 (100)	1900	400	0
20	F-HN+Lip (CCS)	300/1	704±525 (100)	30000	5000	500

In groups 18-20 preformed liposomes were mixed with the soluble antigen.

Table 3B – Lung levels of HI, IgG1, IgG2a and IgA

No.	Vaccine (n=5)	Lipid/HN w/w ratio	Lung HI	IgG1	IgG2a	IgA
1	F-HN		0	0	0	0
2	Lip (DOTAP)-HN	300/1	40	600	85	30
3		100/1	40	500	20	0
4		30/1	30	250	35	0
5		10/1	20	250	0	0
6		3/1	10	20	0	0
7	Lip (DMTAP)-HN	300/1	0	5500	200	1200
8		100/1	0	7000	350	0
9		50/1	0	4500	250	0
10		30/1	0	1500	110	0
11		10/1	0	500	0	0
12	Lip (CCS)-HN	300/1	80	12500	3000	20000
13		100/1	80	7000	5500	65000
14		50/1	40	5500	900	20000
15		30/1	0	1500	200	0
16		10/1	0	500	200	0
17	F-HN+CT (1μg)	-	80	45000	2250	3000
18	F-HN+Lip (DOTAP)	300/1	0	6000	500	1200
19	F-HN+Lip (DMTAP)	300/1	0	3750	225	1500
20	F-HN+Lip (CCS)	300/1	80	35000	3000	80000

Table 3C – Spleen INFγ levels (pg/ml)

No.	Vaccine (n=5)	Lipid/HN w/w ratio	Spleen IFNγ (pg/ml)
1	F-HN		7430
2	Lip (DOTAP)-HN	300/1	9780
3		100/1	42220
4		30/1	20440
5		10/1	20400
6		3/1	27780
7	Lip (DMTAP)-HN	300/1	Not done
8		100/1	
9		50/1	
10		30/1	
11		10/1	
12	Lip (CCS)-HN	300/1	
13		100/1	
14		50/1	
15		30/1	
16		10/1	
17	F-HN+CT (1μg)	-	
18	F-HN+Lip (DOTAP)	300/1	
19	F-HN+Lip (DMTAP)	300/1	
20	F-HN+Lip (CCS)	300/1	

The superiority of Lip CCS-HN vaccine over the other vaccine formulations is again seen as reflected by the high levels of serum and lung IgG2a and IgA antibodies (groups 12-16). Interestingly, simple mixing of soluble antigen with preformed liposomes generated very potent vaccines (groups 18-20) that are equal to liposomes encapsulating the antigen. This suggests that real encapsulation of the antigen may not be necessary for the adjuvanticity of the cationic assemblies/liposomes.

In a further experiment the effect of cholesterol on the immunogenicity of the HN-loaded liposomes was tested. **Tables 4A-4C** show the results of this experiment, indicating that the addition of Chol slightly reduced the systemic HI response to DOTAP-HN at 2/1 and 4/1 mole ratios (groups 4, 5), but not at a 1/1 mole ratio (group 3), and moderately enhances the overall response to DMTAP-HN at all ratios (groups 7-9) and the local (lung) response CCS-HN at a 1/1 ratio (group 11).

Table 4A - Serum levels of HI, IgG1, IgG2a and IgA antibodies

No.	Vaccine (n=5)	Cat lipid/Chol w/w ratio	Serum HI	IgG1	IgG2a	IgA
1	F-HN	-	0	0	0	0
2	Lip (DOTAP)-HN	1/0	320±0 (100)	15000	450	0
3	Lip (DOTAP:Chol)-HN	1/1	496±295 (100)	15000	450	0
4		2/1	168±216 (100)	7000	800	0
5		4/1	195±111 (100)	15000	250	0
6	Lip (DMTAP)-HN	1/0	320±188 (100)	20000	290	0
7	Lip (DMTAP:Chol)-HN	1/1	672±419 (100)	30000	300	0
8		2/1	576±368 (100)	25000	650	0
9		4/1	608±382 (100)	30000	600	0
10	Lip (CCS)-HN	1/0	2560±1568 (100)	30000	7000	100
11	Lip (CCS:Chol)-HN	1/1	2368±1805(100)	30000	9000	100
12	F-HN+CT (1μg)	-	1664±572 (100)	55000	7000	20

Table 4B – Lung levels of HI, IgG1, IgG2a and IgA antibodies

No.	No.	Vaccine (n=5)	Cat lipid/Chol w/w ratio	Lung HI	IgG1	IgG2a	IgA
1		F-HN	-	0	0	0	0
2		Lip (DOTAP)-HN	1/0	40	900	85	25
3		Lip (DOTAP:Chol)-HN	1/1	40	600	80	30
4			2/1	40	680	180	22
5			4/1	60	720	50	60
6		Lip (DMTAP)-HN	1/0	60	1000	40	0
7		Lip (DMTAP:Chol)-HN	1/1	120	3000	30	15
8			2/1	160	2500	160	200
9			4/1	80	4000	100	150
10		Lip (CCS)-HN	1/0	640	30000	1500	9000
11		Lip (CCS:Chol)-HN	3/2	1280	30000	1900	15000
12		F-HN+CT (1μg)	-	20	10000	1800	1000

Table 4C – Spleen INFγ levels (pg/ml)

No.	No.	Vaccine (n=5)	Cat lipid/Chol w/w ratio	Spleen IFNγ (pg/ml)
1		F-HN	-	7430
2		Lip (DOTAP)-HN	1/0	7480
3		Lip (DOTAP:Chol)-HN	1/1	9780
4			2/1	12870
5			4/1	9330
6		Lip (DMTAP)-HN	1/0	8520
7		Lip (DMTAP:Chol)-HN	1/1	10900
8			2/1	8560
9			4/1	7490
10		Lip (CCS)-HN	1/0	15550
11		Lip (CCS:Chol)-HN	3/2	13780
12		F-HN+CT (1μg)	-	11110

The immunogenicity of CCS-HN vaccine was also evaluated in aged (18 month) C57BL/6 mice following intramuscular (once on day 0) or intranasal (twice, days 0 and 7) administration of 1μg and 2μg, respectively, of subunit (HN) vaccine (derived from A/Panama [H3N2] virus). The lipid assemblies were composed of CCS/cholesterol (3:2 molar ratio) and the lipid/HN w/w ratio was 200:1. As opposed to zero activity of the commercial vaccine, the CCS-HN vaccine evoked high levels of serum HI and IgG2a antibodies (tested at 4 weeks

post vaccination)and lung (tested at 6 weeks post vaccination) IgG2a and IgA antibodies as can be seen in Tables 5A and 5B (the data show mean titers).

Table 5A - Serum levels of HI, IgG1, IgG2a and IgA in aged mice

No.	Vaccine[a] (n=5)	Serum HI	IgG1	IgG2a
1	PBS i.n. x2	0	0	0
2	F-HN i.m. x1	0	15	0
3	F-HN i.n. x2	0	0	0
4	Lip (CCS)-HN i.n. x2	80	130	350

Table 5B - Lung levels of IgG1, IgG2a and IgA in aged mice

No.	Vaccine (n=5)	Lung IgG1	IgG2a	IgA
1	PBS i.n. x2	0	0	0
2	F-HN i.m. x1	0	0	0
3	F-HN i.n. x2	0	0	0
4	CCS-HN i.n. x2	0	180	840

In addition, the induction of cellular responses by the various vaccine formulations was tested. In particular, young mice were immunized i.n. with various cationic liposomal formulations and the splenocyte cellular responses — cytotoxicity, proliferation and IFNγ production – were measured 6 weeks after vaccination. In the experiment, the results of which are shown in **Table 6**, a comparison was made between HN-loaded liposomes (groups 3-10) and free antigen (F-HN) given alone (group 2) or admixed with preformed empty liposomes (groups 11-13). The immunogenicity of Lip (DMTAP)-HN and Lip (CCS)-HN prepared at varying lipid/HN w/w ratios (30/1-300/1) was also determined.

Table 6 – Induction of cellular responses by cationic liposomes administered i.n.

No.	Vaccine	Lipid/HN w/w ratio	% cytotoxicity P815 + peptide	% cytotoxicity P815	Proliferation Δcpm (mean)	IFNγ (pg/ml)
1	PBS	-	6	4	7010	1900
2	F-HN	-	8	5	7700	4500
3	Lip (DMTAP)-HN	300/1	16	13	10960	3500
4		100/1	9	9	12870	5850
5		50/1	3	2	17670	3400
6		30/1	3	2	17920	3050
7	Lip (CCS)-HN	300/1	4	2	20370	8000
8		100/1	21	7	24870	8250
9		50/1	6	3	20980	10650
10		30/1	8	5	11510	3500
11	F-HN + Lip (DOTAP)	300/1	17	4	19390	3400
12	F-HN + Lip (DMTAP)	300/1	17	7	11850	5700
13	F-HN + Lip (CCS)	300/1	16	8	19270	4100

Preferential cytotoxicity against the specific target cells (P815 pulsed with the influenza peptide) was obtained only with CCS-HN at a lipid/HN w/w ratio of 100/1 (group 8) and with all the three preformed liposomes (DOTAP, DMTAP and CCS) co-administered with free antigen. The maximum proliferative response was observed with DMTAP-HN at lipid/HN w/w ratios of 50/1 and 30/1 and with CCS-HN at 300/1, 100/1 and 50/1 ratios. The proliferative and cytotoxic responses elicited by the most efficacious liposomal formulations were 2-3 times greater than those induced by free antigen.

These findings suggest that as compared with the humoral response (Table 3), where the highest levels of all types of antibodies measured were obtained at lipid/HN w/w ratios of 100/1-300/1, lower w/w ratios (e.g. 30/1-100/1) may be optimal for the cellular responses. Moreover, whereas DMTAP-HN elicits a strong humoral response, this formulation is a poor inducer of cytotoxic activity, as compared with CCS-HN. Interestingly, vaccination with mixtures of free antigen with preformed cationic liposomes (all three formulations) in suspension evokes good cellular responses that are similar in magnitude to those induced by the encapsulated antigen. Thus, simple mixing of free antigen with preformed

cationic liposomes may be sufficient to induce both strong humoral (**Table 3A-3C**) and cellular (**Table 6**) responses.

In yet a further experiment, the results of which are shown in **Tables 7A–7C**, a comparison was made between 1 i.m. dose, 1 or 2 i.n. doses and 2 oral doses of a monovalent HN-loaded cationic liposomes comprising DOTAP, DMTAP or CCS with regard to immunogenicity and induction of protective immunity to live virus challenge. In this experiment, the lipid/HN w/w ratio was 300/1 and the cationic lipid/Chol ratio was 1/1 for DOTAP and DMTAP systems and 3/2 for CCS system. Of the three routes, i.n. administration twice generates the strongest humoral and cellular response and protective immunity. Of the 3 formulations, CCS induces the highest response, particularly with regard to IgG2a and IgA antibodies.

Table 7A – Serum levels of HI, IgG1, IgG2a and IgA

No.	Vaccine (n=10)	Route	HI	Serum IgG1	IgG2a	IgA
1	PBS		0	0	0	0
2	F-HN	i.m. x 1	60±37 (70)	1000	40	0
3		oral x 2	0	0	0	0
4		i.n. x 1	0	0	0	0
5		i.n. x 2	0	55	0	0
6	Lip (DOTAP/Chol)-HN	i.m. x 1	424±141 (100)	21000	5500	0
7		oral x 2	0	0	0	0
8		i.n. x 1	40±28 (50)	450	80	0
9		i.n. x 2	409±172 (100)	25000	1300	60
10	Lip (DMTAP/Chol)-HN	i.m. x 1	768±211 (100)	24000	8000	0
11		oral x 2	0	0	0	0
12		i.n. x 1	10±10 (0)	300	60	0
13		i.n. x 2	532±763(100)	10500	380	50
14	Lip (CCS/Chol)-HN	i.m. x 1	864±1100 (100)	25000	10000	0
15		oral x 2	0	0		
16		i.n. x 1	34±50 (20)	1000	30	0
17		i.n. x 2	2289±1576 (100)	25000	20000	400
18	F-HN+CT (1μg)	i.n. x 2	756±650 (100)	21000	15000	20

Table 7B - Lung antibodies

No.	Vaccine (n=5)	Route	Lung HI	IgG1	IgG2a	IgA
1	PBS		0	0	0	0
2	F-HN	i.m. x 1	0	80	0	0
3		oral x 2	0	0	0	0
4		i.n. x 1	0	0	0	0
5		i.n. x 2	0	70	20	0
6	Lip (DOTAP/Chol)-HN	i.m. x 1	40	900	500	0
7		oral x 2	0	0	0	0
8		i.n. x 1	0	50	20	0
9		i.n. x 2	120	10000	1000	350
10	Lip (DMTAP/Chol)-HN	i.m. x 1	20	900	150	0
11		oral x 2	0	0	0	0
12		i.n. x 1	0	35	20	0
13		i.n. x 2	240	20000	700	2200
14	Lip (CCS/Chol)-HN	i.m. x 1	60	3500	900	0
15		oral x 2	0	0	0	0
16		i.n. x 1	0	120	0	35
17		i.n. x 2	360	30000	5000	20000
18	F-HN+CT (1μg)	i.n. x 2	240	22000	2500	1800

Table 7C – Cellular response and protective immunity

No.	Vaccine (n=5)	Route	Spleen Δcpm (mean)	IFNγ (pg/ml)	Lung Virus titer (log 10)
1	PBS		1641	0	7
2	F-HN	i.m. x 1	1909	0	4
3		oral x 2	2253	0	ND
4		i.n. x 1	669	0	ND
5		i.n. x 2	2813	0	5
6	Lip (DOTAP/Chol)-HN	i.m. x 1	3452	3300	0
7		oral x 2	0	1150	ND
8		i.n. x 1	482	1900	ND
9		i.n. x 2	8391	3200	0
10	Lip (DMTAP/Chol)-HN	i.m. x 1	5632	0	1
11		oral x 2	553	0	ND
12		i.n. x 1	1277	0	ND
13		i.n. x 2	7331	3150	0
14	Lip (CCS/Chol)-HN	i.m. x 1	6196	5750	0
15		oral x 2	476	550	ND
16		i.n. x 1	1705	6250	ND
17		i.n. x 2	4912	15500	0
18	F-HN+CT (1μg)	i.n. x 2	1933	5650	0

In the experiment described in Tables 8–10, a commercial **trivalent** vaccine was tested and a comparison was made between a single CCS-based vaccine dose (using 2 or 4 μg of antigen [HN] of each viral strain) and two vaccine doses (2 μg/strain/dose), given at 3, 7 or 14 day intervals between administrations. The lipid assemblies were composed of CCS/Chol (cholesterol) at a 3/2 mole ratio, and the lipid/HN w/w ratio was 100/1 for all formulations. As controls, the standard trivalent commercial vaccine (HN) was administered either alone or combined with 1μg cholera toxin (CT), used as a mucosal adjuvant. Sera, lung homogenates and nasal washes were tested 5-6 weeks after the first vaccine dose for HI antibodies (**Table 8**), as well as for antigen-specific IgG1, IgG2a, IgA and IgE antibodies (**Table 9**). In addition, 5 mice from selected groups were challenged i.n. with live virus (using the mouse adapted reassortant X-127 virus) and protection was assessed by quantifying lung virus titer 4 days later (**Table 10**).

As opposed to the poor or no immunogenicity of the commercial flu vaccine (HN) (groups 2-6), CCS/Chol-flu vaccine induced high titers of all types of antibodies tested (except for IgE which was undetected), especially against the two A virus strains (groups 8-11; Tables 8, 9). For the 2-dose regimen, a 1-week interval appears to be the optimal (gr. 10). For the single dose regimen, 4 μg antigen, but not 2 μg (gr. 8 vs. gr. 7), induced high titers of serum HI, IgG1 and IgG2a antibodies and lung IgG1 antibodies. However, in comparison with the 2-dose regimen, the 1-dose regimen did not elicit lung IgG2a and IgA antibodies nor nasal antibodies (Table 9).

In the protection assay (Table 10), the CCS-flu vaccine administered i.n. either once (4 μg) or twice (2 μg/dose) afforded full protection against viral infection (6 log reduction in lung virus titer) whereas the standard vaccine reduced virus titer by only 0.5-1 log. Thus, although the single dose regimen with the CCS-flu vaccine is inferior to the two-dose regimen for certain antibody isotypes, the two regimens provide a similar degree of protection.

In this experiment, we also compared CCS alone to CCS/Chol as the vaccine carrier, and found no difference in immunogenicity between the two formulations (data not shown). Another formulation modification was the reduction of the size of the CCS/Chol lipid assemblies (diameter 0.05-5 μm) by extrusion (diameter ≤0.02 μm). Antibody titers induced by the extruded vaccine were 50-80% lower than those produced by the non-extruded vaccine (data not shown). Thus, unsized CCS lipid assemblies, with or without cholesterol, are highly efficient as a vaccine carrier for trivalent flu vaccine.

Table 8 – Elicitation of hemagglutination inhibition (HI) antibodies following intranasal vaccination with trivalent influenza vaccine, free and in CCS lipid assemblies, administered once or twice at various time intervals to young (2 mo.) BALB/C mice

No.	Vaccine[a] (n=5)	Dosing	days	Mean HI titer (% seroconversion)[b]					
				A/New Caledonia		A/Panama		B/Yamanashi	
				serum	lung	serum	lung	serum	lung
1	None (PBS)	x2	0,7	0	0	0	0	0	0
2	F-HN	2 μg x1	0	0	0	0	0	0	0
3		4 μg x1	0	0	0	0	0	0	0
4		2 μg x2	0, 3	0	0	0	0	0	0
5		2 μg x2	0, 7	0	0	0	0	0	0
6		2 μg x2	0, 14	0	0	0	0	0	0
7	Lip (CCS/Chol)-HN	2 μg x1	0	0	0	0	0	0	0
8		4 μg x1	0	336 (100)	40	328 (100)	40	52 (80)	0
9		2 μg x2	0, 3	544 (100)	80	408 (100)	40	52 (80)	0
10		2 μg x2	0, 7	544 (100)	80	544 (100)	120	88 (100)	0
11		2 μg x2	0, 14	480 (100)	60	368 (100)	40	80 (80)	0
12	F-HN + CT (1 μg)	2 μg x2	0, 7	608 (100)	80	664 (100)	120	84 (80)	0

[a] Mice were immunized with Fluvirin® 2003/2004 trivalent subunit vaccine preparation consisting of A/New Caledonia/20/99 (H1N1)-like, A/Moscow/10/99 (H3N2)-like and B/Hong Kong/330/2001-like, either free (F-HN) or incorporated into CCS/Chol (3/2 mole ratio) lipid assemblies (0.6 mg for groups 7, 9, 10, 11; 1.2 mg for group 8).

[b] Serum HI titer was determined on individual mice 35 days after the first vaccine dose. Lung (pooled) HI titer was tested on day 42.

In parentheses - % of mice with HI titer ≥40. 0 denotes HI titer <20.

Table 9 – Elicitation of serum, lung and nasal antigen-specific IgG1, IgG2a and IgA antibodies following intranasal vaccination with trivalent influenza vaccine, free and in CCS lipid assemblies, administered once or twice at various intervals to young (2 mo.) BALB/c mice

No.	Vaccine[a] (n=5)		Dosing days	Mean antibody titer							
				Serum		Lung Homogenate			Nasal wash		
				IgG1	IgG2a	IgG1	IgG2a	IgA	IgG1	IgG2a	IgA
1	None (PBS)	x2	0,7	0	0	0	0	0	0	0	0
2	F-HN	2 μg x1	0	0	0	0	0	0	0	0	0
3		4 μg x1	0	320	90	1500	0	0	0	0	0
4		2 μg x2	0, 3	0	0	0	0	0	0	0	0
5		2 μg x2	0, 7	0	0	0	0	0	0	0	0
6		2 μg x2	0, 14	40	0	0	0	0	0	0	0
7	Lip (CCS/Chol)-HN	2 μg x1	0	300	0	600	0	0	0	0	0
8		4 μg x1	0	12000	4500	13000	0	0	0	0	0
9		2 μg x2	0, 3	15000	10000	15000	2500	3500	0	10	0
10		2 μg x2	0, 7	15000	12000	14000	2500	9000	200	30	100
11		2 μg x2	0, 14	13000	5500	12000	1800	3000	50	0	0
12	F-HN+CT (1 μg)	2 μg x2	0, 7	21000	15000	20000	2500	2000	250	30	45

[a] See table 8 for experimental details. Samples were pooled and tested by ELISA against the 3 viral strains (pooled HN) 42 days after the first vaccine dose. 0 denotes titer <10.

Table 10 – Protection of young BALB/c mice against viral challenge following intranasal vaccination with trivalent influenza vaccine, free and in CCS lipid assemblies

No.	Vaccine[a] (n=5)	Dosing days	Lung virus titer (log 10)[b]
1	None	-	6
2	F-HN 4 μg x1	0	5.5
3	F-HN 2 μg x2	0, 7	5
4	Lip (CCS/Chol)-HN 4 μg x1	0	0
5	Lip (CCS/Chol)-HN 2 μg x2	0, 7	0
6	F-HN 2 μg + CT (1 μg) x2	0, 7	0

[a] See table 8 for experimental details. In groups 4, 5 the lipid/HN w/w ratio was 100/1.

[b] The mice were infected intranasally 42 days after the first vaccine dose, using ~10^6 egg infectious dose 50% (EID 50) of the mouse-adapted reassortant X-127 virus (A/Beijing/262/95 [H1N1] x X-31 [A/Hong Kong/1/68 x A/PR/8/34). Lungs were harvested 4 days later, homogenized, serially diluted, and injected into the allantoic sac of 10 d. fertilized chicken eggs. After 48 h at 37°C and 16 h at 4°C, 0.1 mL of allantoic fluid was removed and checked for viral presence by hemagglutination.

In the experiment described in Tables 11 and 12, the trivalent-flu vaccine was formulated with the CCS/Chol lipid assemblies using varying amounts of the HN antigens and the lipid. In this experiment the vaccines were prepared with: (a) varying amounts of the antigen (0.25 - 2 μg per viral strain) and of the lipid (0.075 - 0.6 mg), keeping the lipid/HN w/w ratio constant at 100/1; (b) graded amounts of the antigen (0.25 - 2 μg) and a constant amount of the lipid (0.6 mg) thereby varying the lipid/HN w/w ratio from 100/1 to 800/1. As can be seen in Table 11 (HI titer) and Table 12 (isotype titers) vaccines prepared at a 100/1 lipid/HN w/w ratio using 2 or 1 μg antigen of each strain and 0.6 or 0.3 mg lipid, respectively, produced high and similar levels of antibodies against the 3 viral strains (groups 2, 3). At lower antigen (0.5, 0.25 μg/strain) and lipid (0.15, 0.075 mg) doses the response decreased markedly (groups 4, 5), particularly the mucosal response (lung, nasal) (Table 12). When a constant dose of lipid was used (0.6 mg), high levels of antibodies were obtained even with the two lower doses of antigen (0.25, 0.5 μg/strain) (groups 6-8). Thus, the amount of the CCS lipid is critical, and with the appropriate lipid dose the antigen dose can be reduced 4-8 fold (from 1-2 μg to 0.25 - 0.5 μg).

Table 11 – Effect of the antigen dose and lipid dose on the induction of HI antibodies following intranasal vaccination with trivalent influenza vaccine formulated with CCS lipid assemblies, administered twice (at 1 week interval) to young (2 mo.) BALB/c mice

No.	Vaccine[a] (n=5)	HN (µg)	Lipid (mg)	Lipid/HN w/w ratio	Mean HI titer (% seroconversion)					
					A/New Caledonia		A/Panama		B/Yamanashi	
					Serum	Lung	Serum	Lung	Serum	Lung
1	F-HN	2	-	-	0	0	0	0	0	0
2	Lip (CCS/Chol)-HN	2	0.6	100/1	544 (100)	80	544 (100)	120	88 (100)	0
3		1	0.3	100/1	320 (100)	80	544 (100)	160	40 (100)	0
4		0.5	0.15	100/1	416(100)	20	448 (100)	40	32 (100)	0
5		0.25	0.075	100/1	180 (100)	0	100 (100)	20	0	0
6		1	0.6	200/1	672 (100)	80	736 (100)	160	104 (100)	0
7		0.5	0.6	400/1	560 (100)	80	608 (100	160	104 (100)	0
8		0.25	0.6	800/1	512 (100)	80	512 (100)	120	48 (100)	0

[a] See Table 8 for experimental details.

Table 12 – Effect of the antigen dose and lipid dose on the induction of serum, lung and nasal antigen-specific IgG1, IgG2a and IgA antibodies following intranasal vaccination with trivalent influenza vaccine formulated with CCS lipid assemblies, administered twice (at 1 week interval) to young BALB/c mice

	Vaccine[a] (n=5)	HN (µg)	Lipid (mg)	Lipid/HN w/w ratio	Mean antibody titer							
					Serum		Lung homogenate			Nasal wash		
					IgG1	IgG2a	IgG1	IgG2a	IgA	IgG1	IgG2a	IgA
1	F-HN	2	-	-	0	0	0	0	0	0	0	0
2	Lip (CCS/Chol)-HN	2	0.6	100/1	15000	12000	14000	2500	9000	200	30	100
3		1	0.3	100/1	14000	2500	10000	1000	8000	100	0	80
4		0.5	0.15	100/1	15000	1300	8000	1500	4000	0	0	0
5		0.25	0.075	100/1	12000	400	3500	400	2500	0	0	0
6		1	0.6	200/1	20000	15000	12000	2500	8000	200	15	80
7		0.5	0.6	400/1	15000	14000	15000	5000	15000	150	35	100
8		0.25	0.6	800/1	15000	9000	21000	2500	13000	250	25	90

[a] See Tables 8, 9 for experimental details.

In a further experiment, the subunit flu vaccine, either free (HN) or associated with the CCS/Chol lipid assemblies (Lip HN), was tested for its ability to induce HI antibodies cross-reacting with various influenza A and B substrains that were not included in the vaccine. The data shown in **Table 13** indicate that intranasal (i.n.) and intramuscular (i.m.) vaccination, administered once or twice, with either a monovalent or trivalent CCS-based influenza vaccine elicits high serum titers of HI antibodies directed against the immunizing strains, as well as HI antibodies cross-reacting with several A/H1N1, A/H3N2 and B strains that were circulating in the years 1986-1999 and were not included in the vaccine. Slightly lower HI titer were found after a single i.n. vaccine dose (gr. 6 vs. gr. 7). Lung homogenate HI titers (gr. 4, 8) were lower than the corresponding serum titers. Thus, parenteral or intranasal vaccination with the CCS-based vaccine may afford protection against a wide spectrum of A and B viral strains. Such antigenic variants may emerge during a flu epidemic/pandemic as a result of antigenic drift. In contrast, the standard commercial vaccine administered i.n. (gr. 1, 5) was totally ineffective in inducing antibodies against both the homologous and the heterologous strains.

Table 13 – Induction of strain cross-reactive HI antibodies following intranasal or intramuscular vaccination of young BALB/c mice with CCS-based monovalent and trivalent influenza vaccine

No.	Vaccine[a]	Vaccine strains	Sample tested	Mean HI titer against: A/H1N1				A/H3N2				B	
				New Caledonia/ 20/99	Beijing/ 262/95	Texas/ 36/91	Singapore/ 6/86	Panama/ 2007/99	Sydney/ 5/97	Nanchang/ 333/95	Johannesburg/ 33/94	Yamanashi/ 166/98	Harbin/ 07/94
1	HN 2 µg x2 i.n.	A/New Caledonia	serum	**0**	0	0	0	**0**	0	0	0	0	0
2	Lip HN 2 µg x2 i.n.		serum	**1280**	1280	1280	240	**0**	0	0	0	0	0
3	Lip HN 1 µg x1 i.m.		serum	**640**	640	320	40	**0**	0	0	0	0	0
4	Lip HN 2 µg x2 i.n.		lung homo-genate	**320**	240	240	20	**0**	0	0	0	0	0
5	HN 2 µg x2 i.n.	A/New Caledonia, A/Panama, B/Hong Kong	serum	**0**	0	0	0	**0**	0	0	0	0	0
6	Lip HN 4 µg x1 i.n.		serum	**320**	80	120	0	**320**	320	120	120	60	120
7	Lip HN 2 µg x2 i.n.		serum	**480**	120	240	20	**640**	640	120	120	80	320
8	Lip HN 2 µg x2 i.n.		lung homo-genate	**80**	80	40	0	**120**	80	0	0	0	40
9	HN 2 µg + CT 1 µg x2 i.n.		serum	**480**	240	120	40	**480**	480	120	120	80	240

[a] Pooled sera and lung homogenate obtained 5 weeks after vaccination were tested for HI antibodies. For experimental details, see Table 8. The lipid (Lip) assemblies were composed of CCS/Chol (3/2 mole ratio) and the lipid/HN w/w ratio was 300/1 in groups 2-4 and 100/1 in groups 6-8. Except for groups 3 and 6, the two vaccine doses were spaced 1 week apart. In bold, antibody titers against the immunizing strains. 0 denotes HI titer <10.

Biodistribution of anionic and cationic liposomes loaded with HN and administered intranasally

In a biodistribution experiment, 3 formulations of lipid assemblies: DMPC/DMPG (anionic), DOTAP/Chol (cationic) and CCS/Chol (cationic), either empty or loaded with the influenza HN antigens, were administered intranasally (200μg lipid, 2 μg antigen per mouse) into BALB/c mice. The fluorescently labeled lipid was then traced in the homogenates of various tissues over a period of 24 h (at 1, 5, and 24 hours post administration).

As can be seen in the following Table 14 and in **Fig 2A-2F**, after 1 and 5 hours there was 75-100% recovery of the administered lipid of all the three formulations tested. This recovery however dropped significantly at 24 hours in all formulations except for the CCS formulation. The CCS formulation containing the HN antigens displayed the longest retention (>24h.) in the 3 target organs (nose, lungs, GI tract) while there was no lipid accumulation in the brain and no significant accumulation in the other organs tested (liver, kidneys, heart, spleen).

Table 14: Recovery at 1, 5, and 24 hours of fluorescently labeled lipid assemblies administered intranasal

	% Recovery (of total lipid administered)		
Lipid assembly formulation	**1 hour**	**5 hours**	**24 hours**
DMPC/DMPG (empty)	100.2	99.3	26.9
DMPC/DMPG:HN	100.2	99.9	8.3
DOTAP/Chol (empty)	107.0	75.1	8.1
DOTAP/Chol:HN	99.9	106.4	6.7
CCS/Chol (empty)	99.6	96.9	74.2
CCS/Chol:HN	101.1	101.5	94.5

When ^{125}I-labeled HN was used, its biodistribution resembled that of the fluorescent lipid (data not shown). This long retention of the CCS vaccine

components in the respiratory and GI tracts may explain, in part, its superior immunogenicity over the other liposomal formulations. This is exhibited in the following study in which the antigen component of the vaccine was traced. HN proteins were labeled with ^{125}I and administered intranasally either free or associated with one of the lipid formulations used in the fluorescent biodistribution experiments. Radioactivity of the various tissues was determined at 1, 5 and 24h post instillation.

Table 15 teaches that recovery of the antigen was high in this experiment as well. As can be seen in **Fig. 3A-3D**, the biodistribution pattern of the ^{125}I-labeled HN is similar to that of the lipid (**Fig. 2A-2F**), further establishing that: (a) there is indeed an *in vivo* association between the HN-proteins and the lipid assemblies, and (b) the prolonged retention in the nose of the antigen when associated with the cationic lipid assemblies may be due to the cationic lipid assemblies and not an inherent property of the HN proteins, since there is no HN retention when the protein is administered by itself in soluble form.

Also this experiment may teach that there is no HN protein accumulation in the brain when administered alone or associated with lipid-assemblies (a major safety concern with intranasal vaccination). Since the radioactive tracing method is much more sensitive than the fluorescent method, this result is more confidently based.

Table 15: Recovery of ^{125}I labeled HN administered intranasally either alone or associated with lipid assemblies at 1, 5 and 24 hours

	Recovery (% of total injected)		
Lipid assembly formulation	**1 hour**	**5 hours**	**24 hours**
HN	77	48	17
Lip (DMPC:DMPG) HN	88	50	26
Lip (DOTAP:Chol) HN	105	58	32
Lip (CCS:Chol) HN	100	74	41

In an attempt to test if the protein and lipid are retained and/or cleared by similar or different kinetics in the various tissues, another analysis of the data was performed, where the ratio between the % antigen retention (of the total dose administered) and % lipid retention in the various tissues at various time points was determined. When the ratio is constant and ≅1, it means that both components were similarly retained in the same organ, while when this ratio is either larger or smaller than 1 it suggests that the clearance kinetics of each component was different, and one component was cleared faster than the other.

As can be seen in Table 16 below, the only ratio that remained constant with time was that of CCS/Chol-HN in the nose (ratio = ~0.45). This suggests that: (a) the high retention of the antigen in the nose with CCS and DOTAP is in correlation with the level of association and due to the binding of these formulations to the nasal mucosa, in contrast to DMPC/DMPG; and (b) while the other formulations' components dissociate in the body and are cleared at different rates, the CCS-HN based formulation was stable, especially in the nose, and this may contribute to the enhanced immunogenicity seen with the CCS-based vaccines.

Table 16 Retention of the lipid and HN antigen after i.n. administration

		Lip (DMPC:DMPG) HN			Lip (DOTAP:Chol) HN			Lip (CCS:Chol) HN		
		1 h	5 h	24 h	1 h	5 h	24 h	1 h	5 h	24 h
HN	nose	9%	4%	2%	38%	16%	2%	41%	14%	12%
	lungs	30%	3%	4%	19%	4%	12%	24%	21%	11%
	GI	35%	32%	11%	33%	27%	10%	22%	28%	8%
	recovery:	88%	50%	26%	105%	58%	32%	100%	74%	41%
lipid	nose	0%	0%	0%	46%	56%	0%	88%	30%	25%
	lungs	67%	80%	5%	38%	3%	3%	12%	35%	14%
	GI	33%	20%	4%	16%	47%	3%	1%	37%	55%
	recovery:	100%	100%	8%	100%	106%	7%	101%	102%	95%
HN/lipid ratio	nose	-	-	-	0.82	0.29	-	0.47	0.45	0.47
	lungs	0.44	0.03	0.85	0.50	1.29	3.56	2.01	0.60	0.80
	GI	1.07	1.56	2.96	2.12	0.57	2.86	16.08	0.76	0.15
	recovery:	0.88	0.50	3.12	1.05	0.55	4.78	0.99	0.73	0.44

The values show % retention of the total lipid or HN protein administered.

Preliminary safety study of the intranasal flu vaccine

Toxicity (local, systemic) is a major concern with both i.m. and i.n. vaccines and therefore a pilot toxicity study was studied. Cationic lipid formulations (DMTAP, DOTAP, CCS-based) loaded with the influenza antigens hemagglutinin + neuraminidase (HN) were administered i.n. (twice, spaced 1 week apart) to mice (n=4/group), and blood counts (total, differential), blood chemistry and histological examination (nose, lung sections) were performed 72 hours later. The mice showed no apparent signs of any toxicity. Blood counts and blood chemistry were within the normal range, and, as expected, minimal-mild inflammatory response was seen in the nose and lungs of mice treated with the cationic formulations. A similar, albeit less pronounced, inflammatory response was also seen in some mice treated with saline alone or with the non-encapsulated antigen.

Immunomodulatory activity of CCS-flu vaccine in mice

In these experiments, mice were injected i.p. with various liposomal formulations (composed of DMPC, DMPC/DMPG, DOTAP/Chol, CCS/Chol), 0.5-1 mg lipid, with or without the HN antigens. The mice were either untreated or i.p. injected with thioglycollate (TG, to increase macrophage production) 2 days before the injection of the liposomal formulations. Peritoneal cells were harvested 24-48 h. after administration of the liposomes and used as such or after 4 h. adsorption at 37°C to plastic dishes and removal of the non-adherent cells. In other experiments, peritoneal cells were harvested from TG treated mice and incubated with the liposomal formulations for 24-48h. The cells were tested by flow cytometery for the expression of MHC II and the co-stimulatory molecules CD40 and B7. The supernatants were tested for the cytokines interferon γ (IFN γ), tumor necrosis factor α (TNF α) and interleukin 12 (IL-12), and for nitric oxide (NO).

All the cationic formulations (CCS/Chol, DOTAP/Chol, DMTAP/Chol)

upregulated the expression of B7 and CD40 more than the other formulations (DMPC [neutral], DMPC/DMPG [anionic]) and induced higher levels of IFN γ and IL-12. In some cases the CCS/Chol formulation was more effective than the other cationic formulations. No significant levels of TNF α and NO were induced by any of the formulations. The enhanced expression of co-stimulatory molecules on antigen presenting cells and the induction of IL-12 and IFNγ by the cationic formulations can explain, in part, the greater adjuvant activity of these formulations. These findings combined with the long retention of the CCS-flu vaccine in the respiratory tract (**Fig. 2C** and **2F** and **Fig. 3A-3D**) after intranasal administration may explain why CCS is such an efficient mucosal vaccine carrier/adjuvant.

HEPATITIS A VIRUS (HAV)

In addition to influenza, the immune enhancing potential of CCS lipid assemblies was also tested for HAV vaccine administered by the intranasal (i.n.) and the intrarectal (i.r.) routes.

HAV vaccine (Aventis Pasteur), 10 EU (~1.5 μg protein), was administered twice at a 2-week interval and the response was tested by the ELISPOT technique 3 weeks after the second vaccine dose. CpG-ODN, used as a mucosal adjuvant, was given at 10 μg/dose. The HAV-CCS lipid assemblies were prepared as described above for the influenza vaccine (Table 1).

The data presented in Table 17 show that whereas the commercial HAV vaccine failed to induce an IgA response in both tissues (lamina propria, Peyer's patches) tested, and by both administration routes (i.n., i.r.), the vaccine formulated with either CCS or CpG-ODN generated a significant response in most cases. The combination of HAV-CCS lipid assemblies and CpG-ODN resulted in a synergistic response in all cases. Thus, CCS lipid assemblies alone, and particularly in combination with CpG-ODN, are also effective as a carrier/adjuvant for mucosal vaccination against HAV.

Table 17 – Induction of IgA antibodies following intranasal (i.n.) or intrarectal (i.r.) vaccination of BALB/c mice with hepatitis A virus (HAV) vaccine, alone and in combination with CCS lipid assemblies and/or CpG-ODN

Vaccine	Mean no. of IgA AFC/10^6 cells in:			
	Lamina propria		Peyer's patches	
	i.n.	i.r.	i.n.	i.r.
HAV alone	0	0	0	0
HAV-CCS	12	27	0	1
HAV + CpG-ODN	16	22	0	14
HAV-CCS + CpG-ODN	139	68	28	23

AFC – antibody-forming-cells

C. BOTULINUM

In a further experiment, Mice were immunized i.n. with 0.4 μg dose of a commercial C. botulinum toxoid (as a model for bioterror agent, Uruguay, alum free) and antibody titers were tested by ELISA 4 weeks after the second vaccine dose.

The results of an experiment with C. botulinum toxoid are summarized in Table 18, which shows the superiority of the CCS-toxoid formulation over the standard vaccine following i.n. instillation, particularly with regard to the IgA levels in the small intestine and feces. Such Abs are expected to neutralize the toxin upon oral exposure. Mice immunized i.n. with the vaccine alone did not produce IgA.

Table 18 – Induction of IgG1, IgG2a and IgA antibodies in BALB/c mice vaccinated intranasally (twice, 1 week apart) with free or CCS-associated *Clostridium botulinum* toxoid (CBT)

Vaccine[a] n=10	Mean antibody titer					
	Serum		Small intestine			Feces
	IgG1	IgG2a	IgG1	IgG2a	IgA	IgA
CBT	0	0	1000	180	0	0
CCS-CBT	400	24	1600	0	1800	1800

MATERIALS

Chemistry

Synthesis of N-palmitoyl D-erythro sphingosyl-1-carbamoyl spermine (CCS)

(i) N-palmitoylsphingosine (1.61g, 3mmol) was dissolved in dry THF (100ml) with heating. The clear solution was brought to room temperature and N,N'-disuccinimidyl carbonate (1.92g, 7.5 mmol) was added. DMAP (0.81g, 7.5 mmol) was added with stirring and the reaction further stirred for 16 hours. The solvent was removed under reduced pressure and the residue re-crystallized from n-heptane yielding 1.3g (68%) of disuccinimidylceramidyl carbonate as white powder m.p. 73-76°C.

(ii) Spermine (0.5g, 2.5 mmol) and the disuccinimidylceramidyl carbonate (0.39g, 0.5 mmol) were dissolved in dry dichloromethane with stirring and then treated with catalytic amount of 4-dimethylamino pyridine (DMAP). The solution was stirred at room temperature for 16 hours, the solvent evaporated and the residue treated with water, filtered and dried in vacuo, giving 0.4g (82%) of crude material which was further purified by column chromatography on Silica gel, using 60:20:20 Butanol: AcOH:H_2O eluent.

(iii) For obtaining a quaternary amine within the compound, the product of step (ii) may be methylated with DMS or CH_3I.

The structure of CCS was confirmed by ^{1}H- and ^{13}C-NMR spectrometry (data not shown). Detailed description of the analysis is described in co-pending International patent application No. _____.

Other synthetic procedures

Similarly to the above procedure, the following procedures may be applied:

Synthesis of linear monosubstituted ceramde-spermine conjugate as depicted in Fig. 1A

An equivalent of a ceramide is reacted with 2.5 equivalents of disuccinimidyl carbonate in the presence of DMAP to obtain the corresponding 1,3-di-O-succinimidyl derivative is obtained.

The disuccinimidyl derivative though obtained is reacted with an equivalent of spermine at room temperature using catalytic amount of DMAP to obtain the 3- monosubstituted ceramide-spermine conjugate of Fig. 1B.

Synthesis of linear disusbstituted ceramide-spermine conjugate as depicted in Fig. 1B

An equivalent of 1,3-di-O- succinimidyl sphinogid derivative prepared as described above is reacted with 2.5 equivalents of spermine at 80° in the presence of catalytic amounts of DMAP. The 1,3-disubstituted CCS is though obtained.

Synthesis of linear disusbstituted ceramide - branched spermine conjugate as depicted in Fig. 1C

An equivalent of 1,3-di-O- succinimidyl ceramide derivative prepared as described above is reacted with 2.5 equivalents of *alpha-omega* di protected spermine at 80° in the presence of catalytic amounts of DMAP.

The protection is removed and the 1,3-"branched" disubstituted ceramide-spermine conjugate is obtained.

Synthesis of linear disusbstituted ceramide – cyclic spermine conjugate as depicted in Fig. 1D

An equivalent of 1,3-di-O-succinimidyl ceramide derivative prepared as described above is reacted with 0.75 equivalents of spermine at 80°C in the presence of catalytic amounts of DMAP.

Influenza antigens

A monovalent subunit antigen preparation derived from influenza A/New Caledonia/20/99-like (H1N1) strain was generously provided by Drs. Gluck and Zurbriggen, Berna Biotech, Bern, Switzerland. This preparation (designated herein HN) comprised of 80-90% hemagglutinin, 5-10 wt% neuraminidase and trace amounts of NP and M1 proteins. A commercial trivalent subunit vaccine (Fluvirin®) for the 2003/2004 season containing HN derived from A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2) and B/Shangdong/7/97 was obtained from Evans Vaccines Ltd., Liverpool, UK. This vaccine was concentrated ~x8 (Eppendorf Concentrator 5301, Eppendorf AG, Hamburg, Germany) prior to encapsulation. A whole inactivated virus was used in some experiments for *in vitro* stimulation.

Lipids

The phospholipids (PL) dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), and dioleoyl phosphatidylethanolamine (DOPE) are from Lipoid GmbH, Ludwigshafen, Germany. In addition to DMPC (neutral) and DMPC/DMPG (9/1 mole ratio, anionic) liposomes, 6 formulations of cationic liposomes/lipid assemblies were prepared. The monocationic lipids dimethylaminoethane carbamoyl cholesterol (DC-Chol), 1,2-distearoyl-3-trimethylammonium-propane (chloride salt) (DSTAP), dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), and dimyristoyl-3-trimethylammonium-propane (chloride salt) (DMTAP) are from Avanti Polar Lipids (Alabaster, AL, USA). The monocationic lipid

dimethyldioctadecylammonium bromide (DDAB) and cholesterol (Chol) are from Sigma. The novel, proprietary polycationic sphingolipid N-palmitoyl D-erythro sphingosyl carbamoyl-spermine (acetate salt) (ceramide carbamoyl-spermine, CCS) is from Biolab Ltd., Jerusalem, Israel. Where indicated, the helper lipids (DOPE, Chol) were used at a lipid/helper ratio of 1/1 to 4/1 mole ratio.

Mice

Specific pathogen-free (SPF) female BALB/c mice, 6-8 weeks old, and C57BL/6 mice, 18 month-old, were used (5-10 per group). Animals were maintained under SPF conditions.

METHODS

Encapsulation of influenza antigens in liposomes/lipid assemblies

HN antigens (see above) were encapsulated in large (mean diameter 0.1 – 5 μm) heterogeneous (unsized) vesicles. The following procedure was used routinely for the preparation of all vaccine formulations. Lipids (10-30 mg) were dissolved in 1 ml tertiary butanol, then sterilized by filtration (GF92, Glasforser, Vorfilter no. 421051, Schleicher & Schuell, Dassel, Germany). The sterile lipid solution was frozen at -70°C, then lyophilized for 24 h to complete dryness. The dried lipids could be stored at 4°C for >2 years without significant (<5%) lipid degradation or loss of "encapsulation" capability. Upon need, the lipid powder was hydrated with the antigen solution (in PBS pH 7.2) at a lipid:antigen (protein) w/w ratio of 3/1 to 800/1. The antigen solution was added stepwise in increments of 20-50 μl and vortexed vigorously after each addition, up to a final volume of 0.5-1 ml. In some experiments, the dried lipids were hydrated with PBS and the preformed "empty" lipid assemblies were mixed with the antigen solution. The mixture was vortexed for 1-2 min and used as is within 30-60 min.

To determine "encapsulation" efficiency, two procedures were used, depending on the formulation, resulting in ≥80% separation between the free

antigen and the lipid-associated antigen. For all vaccine formulations, except CCS, the following separation technique was used. The lipid assemblies (1-30 mg lipid) containing the HN antigen (50-100 μg protein) were suspended in 0.5 ml PBS and carefully loaded over 0.5 ml of D_2O (99.9%, Aldrich Chemical Co., Milwaukee, WI, USA). The sample was then centrifuged for 1h at 30°C at 45,000 rpm. The free, non-encapsulated HN precipitates while the assembled (liposomal) HN and protein-free assemblies/liposomes remain in the supernatant. The entire supernatant was collected and the assemblies/liposomes were dissolved by adding 0.2 ml of warm 10% Triton X-100 to both the supernatant and the pellet fractions. Protein concentration in both fractions was determined by the modified Lowry technique. For the CCS formulation, the CCS-HN was suspended in 0.5 ml of PBS-D_2O (1 vol PBS X10 + 9 vol D_2O) then mixed with 0.5 ml of PBS. The mixture was then centrifuged for 10 min at 20°C at 10,000 rpm. The CCS +/- antigen precipitates while the free HN remains in the supernatant. Lipid dissolution and protein determination in both fractions were carried out as described above. In both separation techniques, the overall recovery of the HN antigens was >95%.

Immunization

Free (F-HN) and assembled/liposomal (Lip-HN) vaccines, 0.25-4 μg antigen/strain/dose and 0.075-1.2 mg lipid / dose, were administered either once intramuscularly (i.m., in 30 μl), once or twice intranasally (i.n., in 5-50 μl per nostril) spaced 3, 7 or 14 days apart, or twice orally (in 50 μl) spaced 1 week apart. In all cases, mice were lightly anesthetized with 0.15 ml of 4% chloral hydrate in PBS given intraperitoneally. For oral vaccination mice were treated orally with 0.5 ml of an antacid solution (8 parts Hanks' balanced salt solution + 2 parts 7.5% sodium bicarbonate) 30 min prior to vaccination. Cholera toxin (CT, Sigma, USA), 1 μg/dose, was used in all experiments as a standard mucosal adjuvant for comparison. In two experiments, CpG-ODN (ODN 1018, generously provided by Dr. E. Raz, University of California, San Diego, CA, USA), free and

liposomal, 10 μg/dose, was used as an adjuvant.

Assessment of humoral responses

Sera, lung homogenates and nasal washes were tested, individually or pooled, 4-6 weeks post-vaccination, starting at 1/10 or 1/20 sample dilution. Hemagglutination inhibiting antibodies were determined by the standard hemagglutination inhibition (HI) assay, starting at 1/10 sample dilution. Mice with HI titer ≥40 (considered a protective titer in humans) were defined as seroconverted. Antigen-specific IgG1, IgG2a, IgA and IgE levels were measured by ELISA. The highest sample dilution yielding absorbance of 0.2 OD above the control (antigen + normal mouse serum, OD <0.1) was considered the ELISA antibody titer.

Assessment of cellular responses

Splenocytes obtained at 5-6 weeks after vaccination were tested for proliferative response, IFNγ and IL-4 production, and cytotoxic activity, following *in vitro* stimulation with the antigen. Cultures were carried out at 37°C in enriched RPMI 1640 or DMEM medium supplemented with 5% (for proliferation, cytokines) or 10% (for cytotoxicity) fetal calf serum (FCS), with (for cytotoxicity) or without 5 x 10^{-5}M 2-mercaptoethanol. Cell cultures were performed as follows: (i) Proliferation: 0.5 x 10^6 cells per well were incubated in U-shaped 96-well plates, in triplicate, with or without the antigen (0.5-5 μg per well), in a final volume of 0.2 ml. After 72-96h, cultures were pulsed with 1 μCi ^{3}H-thymidine for 16 h. Results are expressed in Δcpm = (mean counts per minute of cells cultured with antigen) – (mean counts per minute of cells cultured without antigen). (ii) Cytokines: 2.5 x 10^6 to 5 x 10^6 cells per well were incubated in 24-well plates, in duplicate, with or without the antigen (5-10 μg per well), in a final volume of 1 ml. Supernatants were collected after 48-72 h and tested by ELISA for murine IFNγ and IL-4 using the Opt EIA Set (Pharmingen, USA). (iii) Cytotoxicity: Responding splenocytes (2.5x10^6) were incubated as in (ii) for 7

days together with an equal number of stimulating BALB/c splenocytes that had been infected with the X/127(H1N1) influenza virus (see below). For infection, the splenocytes were incubated, with occasional stirring, for 3 h at 37°C in RPMI 1640 medium (without FCS) with 150 hemagglutination units/1x10^6 splenocytes of the virus, followed by washing. Subsequently, the primed effector cells were restimulated for 5 days with infected, irradiated (3,000 rad) splenocytes at an effector/stimulator cell ratio of 1/4 in the presence of 10 IU/ml of rhIL-2. Cytotoxicity was measured using the standard 4 h ^{51}Cr release assay at an effector/target cell ratio of 100/1. The labeled target cells used were unmodified P815 and P815 pulsed for 90 min at 37°C with the HA2 189-199 peptide (IYSTVASSLVL, 20 μg/1x10^6 cells).

Determination of protective immunity

Mice were anesthetized and 25 μl of live virus suspension per nostril, ~10^7 EID 50 (egg-infectious dose 50%), was administered, using the reassortant virus X-127 (A/Beijing/262/95 (H1N1) x X-31 (A/Hong Kong/1/68 x A/PR/8/34), which is infectious to mice and cross-reactive with A/New Caledonia. The lungs were removed on day 4, washed thrice in cold PBS, and homogenized in PBS (1.5 ml per lungs per mouse, referred to as 1/10 dilution). Homogenates of each group were pooled and centrifuged at 2000 rpm for 30 min at 4°C and the supernatants collected. Serial 10-fold dilutions were performed and 0.2 ml of each dilution was injected, in duplicate, into the allantoic sac of 11-day-old embryonated chicken eggs. After 48 h at 37°C and 16 h at 4°C, 0.1 ml of allantoic fluid was removed and checked for viral presence by hemagglutination (30 min at room temperature) with chicken erythrocytes (0.5 wt.%, 0.1 ml). The lung virus titer is determined as the highest dilution of lung homogenate producing virus in the allantoic fluid (positive hemagglutination).

Biodistribution and pharmacokinetics of various fluorescently-labeled lipid formulations and radioactively-labeled HN antigen

Mice were vaccinated once with lissamine-rhodamine labeled lipid assembly formulations either empty or associated with trivalent subunit influenza vaccine (HN) in a volume of 20 µl. After 1, 5 or 24 hours, mice were sacrificed and various organs were removed. The organs were stored at -20 deg overnight, and the next morning homogenized in lysis buffer. 0.2 ml the subsequent homogenate was transferred to eppendorf tubes, 0.8 mL of isopropanol was added, and spun for 15 minutes to release fluorescent probe into the supernatant. 50 uL of the supernatant was loaded onto a 384 black plate and the fluorescence was read (Em: 545, Ex: 596).

In a further assay, 450 µg of trivalent HN vaccine (in 5 mL) were dialysed against DDW (to remove salt) and then concentrated X1000 to 5 µL. The protein was then diluted in 0.1M borate buffer (pH 8.5) to a stock solution of 450 µg in 15 µL. The protein was then labeled with ^{125}I using the Bolton Hunter reagent, according to the manufacturer's instructions. Mice were provided with the ^{125}I-labeled HN (2 µg) and at 1, 5 and 24 hrs, the mice were sacrificed, and various organs (see **Fig 3**) were removed into vials and read in a γ-counter calibrated for ^{125}I.

The invention will now be defined by the appended claims, the contents of which are to be read as included within the disclosure of the specification.